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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/IT92/00081 <b>(22) International Filing Date:</b> 16 July 1992 (16.07.92)  <b>(30) Priority data:</b> RM91/A000546 19 July 1991 (19.07.91) IT  <b>(71) Applicant (for all designated States except US):</b> SORIN BIOMEDICA S.P.A. [IT/IT]; Via Crescentino, I-13040 Saluggia VC (IT).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> ROSA, Carlo [IT/IT]; GRIVA, Silvia [IT/IT]; BONELLI, Fabrizio [IT/IT]; Sorin Biomedica S.p.A., Via Crescentino, I-13040 Saluggia VC (IT).  <b>(74) Agents:</b> BANCHETTI, Marina et al.; Ing. Barzano' & Zandardo Roma S.p.A, Via Piemonte, 26, I-00187 Rome (IT).		<b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> EPITOPES OF THE ENV PROTEIN OF THE HEPATITIS C VIRUS  <b>(57) Abstract</b>  Amino acid sequences having antigenic activity comprised in the sequence of the env protein of the virus HCV are disclosed; synthetic peptides having said sequences show an increased reactivity with anti-HCV sera when cyclized. Different variants of said sequences and nucleotide sequences coding for the same are also disclosed.		

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## EPITOPES OF THE ENV PROTEIN OF THE HEPATITIS C VIRUS

This invention relates to epitopes of the env protein of the hepatitis C virus.

5 More particularly, this invention relates to peptides comprising epitopes of the hepatitis C virus (HCV) localized in the envelope surface viral protein (env), which are capable of reacting with antisera and/or with monoclonal antibodies and it also relates  
10 to the amino acids sequence of said epitopes, as well as to the nucleotide sequence coding for the same.

The documents cited with a numeral reference are listed at the end of this disclosure.

The virus HCV is believed to be responsible for  
15 the hepatitis classified as non-A/non-B (PT-NANB) (1). The existence of an etiological agent for NANB hepatitis has been also proved by Alter et al. (2). The virus has been identified as an RNA virus, of positive polarity, and the genome, in the form of cDNA, has been  
20 wholly cloned and sequenced. From an analysis of the sequence it turned out that the sequence in question consists of about 10,000 ribonucleotides and forms a single reading frame that potentially codes for a single amino acid chain. This same organization is also  
25 present in other viral families such as those of flavivirus and of Pestivirus; however, other structural

characteristics make it uncertain to set forth a precise taxonomic position of HCV.

5 The cloning of a first portion of the genome has been disclosed by Choo, Q.L. et al. (3), and the sequence has been published in the European Patent EP 88310922.5. The regions identified correspond to the so-called nonstructural regions which, in a way similar to that of flavivirus, have been called NS1, NS2, NS3, NS4 and NS5.

10 More recently, structural regions, coding for capsid and for surface proteins, have been cloned and sequenced. Such sequences have been published by Okamoto, H. et al. (4) and in the European Patent application EP 90302866.0.

15 In order to identify immunological markers of HCV infection, large amounts of viral antigens are needed. However, differently from other hepatotropic viruses, such as HBV and HDV, the concentration of HCV in the liver and in the blood is very low and, differently from the virus of hepatitis A (HAV), HCV cannot be  
20 grown in vitro. Therefore it is not available a good natural source of viral antigens.

Accordingly, the preparation of immunological tests requires the availability of synthetic peptides capable of mimicking the immunological activity of  
25 viral antigens. To that aim, the identification of specific protein portions, denominated epitopes,

capable of reacting with antibodies is necessary due to the short length of synthetic peptides. Moreover, it is well known that tests which employ just the epitope of the protein are more sensitive and more accurate.

5 Up to the present time it has been impossible to identify portions with antigenic activity of HCV env protein, capable of reacting with antibodies and, therefore, the env protein or portions thereof has never been employed for immunological tests.

10 It is well known that RNA viruses are characterized by a high frequency of spontaneous mutation. In the case of HCV, variable and hypervariable domains have been identified in the sequences corresponding to the surface proteins (5 and  
15 EP 004191.82A1), possibly related to viral mechanisms of escaping of the immune response. Moreover NANB hepatitis becomes a chronic disease in about 50 % of patients. It is therefore very useful to identify epitopes of surface proteins both for diagnosis and for  
20 prognosis purposes.

The Authors of this invention have identified variable regions with a high antigenic activity of the amino acid sequence of the env protein, and they have found that such regions correspond to epitopes of said  
25 protein. The Authors also have identified some variants of such regions by means of amplification of nucleic

acids from serum samples; among such regions, one is coded by a HCV variant not disclosed before.

The endemic distribution of the different viral variants of HCV virus makes it necessary to prepare assays able to detect epitopes of the different variants.

The Authors have synthesized such epitopes in vitro for immunological assays on serum samples.

The availability of an anti-env marker with serological characteristics such as those of the object of this invention, can lead to more specific tests, which can be particularly employed for anti-HCV screening of blood samples. Indeed, an analysis, carried out by Contreras et al. (6) just employing the test based on the c100 protein gives rise to a remarkable number of false positive results, with no precise identification of the infected samples.

Finally, as tests which employ amplification procedures such as the PCR (polymerase chain reaction) are not exploitable for massive screenings, it is useful to correlate the positive results obtained with the assay realized by the Authors and the results obtained with the PCR.

Accordingly, it is a specific object of this invention an amino acid sequence comprising an epitope of the env protein of the HCV virus, preferably in the

portion from the amino acid 209 to the amino acid 259, according to the numeration as given in (3).

According to some preferred embodiments of this invention, said sequences are included in the following group of sequences: SEQ ID N1, SEQ ID N2 and SEQ ID N3; preferably from the amino acid 13 to the amino acid 46 of SEQ ID N1, more preferably from the amino acid 21 to the amino acid 30 of SEQ ID N1; alternatively from the amino acid 13 to the amino acid 46 of SEQ ID N2, preferably from the amino acid 21 to the amino acid 30 of SEQ ID N2; alternatively from the amino acid 13 to the amino acid 47 of SEQ ID N3, preferably from the amino acid 21 to the amino acid 31 of SEQ ID N3.

It is a further object of the invention a peptide according to any of said amino acid sequences, preferably of synthetic origin, more preferably cyclized by means of reaction of two residues of cysteine.

Again it is an object of this invention a nucleotide sequence coding for an epitope of the env protein, preferably comprised in one of the sequences of the following group: SEQ ID N1, SEQ ID N2, and SEQ ID N3; preferably comprising at least one fragment of 10 nucleotides of SEQ ID N3, more preferably the entire sequence of SEQ ID N3.

This invention will be now disclosed in some working examples of the same, with reference to the following figures, wherein:



- Figures 1A, 1B, and 1C represent the hydr philic profiles respectively of the env 1, env 2 and env 3 variants.

5 EXAMPLE 1 Identification of 3 variants in the region of the env protein and synthesis of the corresponding peptides

10 An investigation carried out by means of nucleic acid amplification procedures from serum samples (PCR, 7) allowed the identification of 3 main variants of the env surface protein to be carried out, said variants being called respectively env 1, env 2, env 3, and comprising the sequences disclosed respectively as SEQ ID N1, SEQ ID N2 and SEQ ID N3.

15 The variant env 1 and env 2 are comprised in viral variants respectively known by those skilled in the art as HCV A1 (american isolate) and HCV J1 (japan isolate). The variant env 3 is coded by a viral variant which is not included in any HCV isolate disclosed up to the present invention, denominated HCV 3. Such  
20 variant differentiates mainly by the insertion of a histidine residue into a region delimited by 2 cysteines, which modifies the hydrophilic profile of the genic product (Figures 1A, 1B and 1C). Such modification is of particular relevance for the analogy with  
25 the transmembrane region of the HIV1 surface protein (8, 9).

Oligopeptides comprising respectively the sequence of the env protein from the amino acid 13 to the amino acid 32 of the SEQ ID N1 (the env 1 variant); the sequence of the env protein from the amino acid 13 to the amino acid 32 of SEQ ID N2 (the env 2 variant); the sequence of the env protein from the amino acid 13 to the amino acid 33 of SEQ ID N3 (the env 3 variant) are synthesized according to Merrifield's method (10), employing as the solid phase a polyamide resin "Pepsin K polyamide Kieselguliz" (Milligen, Novato, California), which had been previously functionalized with ethilendiamine and with 4-(alpha-Fmoc-amino-2',4'-dimethoxybenzyl)phenoxyacetic acid. The amino acids employed for the synthesis are protected on the side chains by tert-butyl groups and on the alpha-amino position with the F-moc group (9-fluoro-methyloxycarbonyl group). The guanidinium group of arginine and the imidazole group of histidine is respectively protected with the substituents consisting of the 2,2,5,7,8-pentamethylchroman-6-sulfonyl and trityl groups. The carboxy group of the amino acids employed is activated by the formation of an ester-type bond with the pentafluorophenyl group. The synthesis is performed with the Milligen 9050 synthesizer (Novato, California) employing the continuous flow method. The removal of protection and the separation of the peptides from the resin are

carried out by treatment with trifluoroacetic acid. The peptide sequence is checked with an automatic microsequencer (Portan Instruments).

#### EXAMPLE 2

##### 5 Cyclization of peptides

Oligopeptides comprising respectively the sequence of the env protein from the amino acid 21 to the amino acid 30 of the SEQ ID N1 (the env 1 variant); the sequence of the env protein from the amino acid 21 to the amino acid 30 of SEQ ID N2 (the env 2 variant); the  
10 sequence of the env protein from the amino acid 21 to the amino acid 31 of SEQ ID N3 (the env 3 variant) are synthesized according to the Example 1.

The cyclization of a fraction of the peptides is carried out in the following way: the peptide is  
15 dissolved in water to a concentration of 0.1 mg/ml. The pH value is adjusted to 7 with 1M  $\text{NH}_4\text{OH}$ . Potassium ferricyanide is then added slowly to the solution (400 mg  $\text{K}_3\text{Fe}(\text{CN})_6$  in 200 ml of water) till persistence of  
20 the yellow colour. The disappearance of the free SH groups is obtained employing the method of Edman (11).

Alternatively the peptide is dissolved at 0.2 mg/ml in distilled/deionized water (Milliq) and the pH is adjusted to pH 8 using a solution of 3M  $\text{NH}_4\text{Cl}$ . The  
25 solution is allowed to stir for four days and the loss of the free sulphide groups is monitored using the Edman titration. Briefly, 24 mg of 5-5'dithio-bis

(2-nitrobenzoic acid) is dissolved in 5 ml of phosphate buffer pH 7. 20  $\mu$ l of this solution is mixed with 1 ml of the peptide solution and the absorbance is read at 412 nm. After four days 96% of the free sulphide groups are disappeared.

### EXAMPLE 3 Immunological assay

In order to determine the immunogenicity of linear and cyclized peptides described in EXAMPLE 2, an ELISA assay is carried out.

The cyclic and linear peptides are dissolved in 50 mM carbonate buffer, pH 9.6 at a concentration of 5  $\mu$ g/ml. 200  $\mu$ l/well of a microtitration plate is dispensed and incubated for 1 hr at 37°C. The overcoating of the wells is performed by coating to the empty wells 300  $\mu$ l of a solution containing 50 mM Tris-HCl pH 7.4 and 0.2% bovine serum albumin (BSA, Sigma, Fraction V). The plates are incubated for 2 hrs at room temperature.

Finally 300  $\mu$ l/well of a solution containing 10% sucrose, 4% polyvinylpyrrolidone and 9% NaCl is added and left for 1 hr at room temperature.

The ELISA assay is performed by dispensing 200  $\mu$ l/well of sera, previously diluted, using a HCV negative serum, as sample diluent. The samples are incubated for 1 hr at 37°C. The plates are then washed five times with a solution containing 0.05% Tween-20, 0.1% BSA in 50mM phosphate buffer pH 7.4 (washing

buffer) and incubated for 1 hr at 37°C with 200  $\mu$ l of a solution containing goat IgG anti-human IgGs, conjugated with horse radish peroxidase (HRP).

5 After five washings with washing buffer the plates are incubated for 30 min with a chromogen-substrate solution (tetramethylbenzidine and 3% hydrogenperoxide). The reaction is stopped with 1N sulphuric acid and the absorbance is read at 450nm.

10 The serum utilized (21) belongs to the panel BBI mixed HCV (Boston Biomedica Inc.). The control HCV negative serum gives constantly values lower than 0.04.

The results are shown in the following Table 1.

15

20

25

Table 1

ELISA assay with serum 21 BBI

		env 1		env 2		env 3	
		cyclic	linear	cyclic	linear	cyclic	linear
5	serum	OD	OD	OD	OD	OD	OD
	dil.	450	450	450	450	450	450
10	1:20	2.150	0.141	1.648	0.114	1.777	0.132
	1:40	1.028	0.093	0.841	0.095	0.980	0.089
	1:80	0.615	0.078	0.512	0.071	0.546	0.076
	1:160	0.243	0.074	0.221	0.064	0.150	0.070
	1:320	0.098	0.061	0.093	0.051	0.090	0.054
15	1:640	0.061	0.060	0.048	-	0.054	0.056

The results show that env 1, env 2 and env 3 peptides are able to react with anti HCV sera. The reactivity is greatly increased when such peptides are made cyclic and therefore have a conformational structure similar to the corresponding region of the whole env protein. The reactivity decreases proportionally with serum dilutions, thus indicating that the reaction is specific.

This invention has been disclosed with specific reference to some preferred embodiments of the same,

but it is to be understood that modifications and/or changes can be introduced by those who are skilled in the art without departing from the spirit and scope of the invention for which a priority right is claimed.

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## LIST OF THE SEQUENCE CHARACTERISTICS

SEQ ID N1

SEQUENCE TYPE: Nucl otid with corresponding peptide

LENGTH OF THE SEQUENCE: 153 base pairs

5 CONFORMATION: single helix

TOPOLOGY: linear

MOLECULAR TYPE: cDNA from genomic RNA

HYPOTHETIC SEQUENCE: no

ANTI-SENSE: no

10 ORIGINAL SOURCE: HCV virus variant A1

EXPERIMENTAL SOURCE: genic library from viral isolate

CHARACTERISTICS: coding for a portion of env protein  
variant env 1

IDENTIFICATION METHOD: experimental

15 PROPERTY: coding sequence

AAC TCG AGC ATT GTG TAC GAG GCT GCC GAC 30

Asn Ser Ser Ile Val Tyr Glu Ala Ala Asp

1 5 10

GCC ATC CTG CAC ACT CCG GGG TGC GTC CCT 60

Ala Ile Leu His Thr Pro Gly Cys Val Pro

11 15 20

TGC GTT CGC GAG GGT AAC GCC TCG AGG TGT 90

Cys Val Arg Glu Gly Asn Ala Ser Arg Cys

21 25 30

TGG GTG GCG ATC ACC CCC ACG GTG GCC ACC 120

Trp Val Ala Ile Thr Pro Thr Val Ala Thr

25 31 35 40

AGG GAT GGC AAA CTC CCC ACA GCG CAC GTT 150

Arg Asp Gly Lys Leu Pro Thr Ala His Val

41 45 50

CGA

Arg

51



SEQ ID N2

SEQUENCE TYPE: Nucleotide with corresponding protein

LENGTH OF THE SEQUENCE: 153 base pairs

CONFORMATION: single helix

5 TOPOLOGY: linear

MOLECULAR TYPE: cDNA from genomic RNA

HYPOTHETIC SEQUENCE: no

ANTI-SENSE: no

ORIGINAL SOURCE: HCV virus variant J1

10 EXPERIMENTAL SOURCE: genic library from viral isolate

CHARACTERISTICS: coding for a portion of env protein  
env 2 variant

IDENTIFICATION METHOD: experimental

PROPERTY: coding sequence

15 AAC TCA AGC ATC GTG TAT GAG GCA GCA GAC 30  
Asn Ser Ser Ile Val Tyr Glu Ala Ala Asp  
1 5 10  
TTG ATC ATG CAC ACC CCC GGG TGC GTG CCC 60  
Leu Ile Met His Thr Pro Gly Cys Val Pro  
11 15 20  
20 TGC GTT CGG GAG AAC AAC CTC TCC CGC TGC 90  
Cys Val Arg Glu Asn Asn Leu Ser Arg Cys  
21 25 30  
TGG GTA GCG CTC ACT CCC ACG CTT GCG GCC 120  
Trp Val Ala Leu Thr Pro Thr Leu Ala Ala  
31 35 40  
25 AGG AAT GTC AGC GTC CCC ACA GCA ACA ATA 150  
Arg Asn Val Ser Val Pro Thr Ala Thr Ile  
41 45 50  
CGA  
Arg

SEQ ID N3

SEQUENCE TYPE: Nucleotide with corresponding protein

LENGTH OF THE SEQUENCE: 156 base pairs

CONFORMATION: single helix

5 TOPOLOGY: linear

MOLECULAR TYPE: cDNA from genomic RNA

HYPOTHETIC SEQUENCE: no

ANTI-SENSE: no

ORIGINAL SOURCE: HCV virus variant 3

10 EXPERIMENTAL SOURCE: genic library from viral isolate

CHARACTERISTICS: coding for a portion of the env  
protein env 3 variant

IDENTIFICATION METHOD: experimental

PROPERTY: coding sequence

15	AAC TCA AGT ATT GTG TAT GAG GCA GCG GAC	30
	Asn Ser Ser Ile Val Tyr Glu Ala Ala Asp	
	1 5 10	
	CTG ATC ATG CAC ACC CCC GGG TGC GTG CCC	60
	Leu Ile Met His Thr Pro Gly Cys Val Pro	
	11 15 20	
20	TGC GTT CGG GAA GGA GAC AAC CAC TCC CGC	90
	Cys Val Arg Glu Gly Asp Asn His Ser Arg	
	21 25 30	
	TGC TGG GTA GCG CTC ACT CCC ACT CTC GCG	120
	Cys Trp Val Ala Leu Thr Pro Thr Leu Ala	
	31 35 40	
25	GCC AGG AAT AGC AGC GTC CCC ACC ACG ACA	150
	Ala Arg Asn Ser Ser Val Pro Thr Thr Thr	
	41 45 50	
	ATA CGA	
	Ile Arg	
	51	

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## CLAIMS

1. An amino acid sequence characterized in that it comprises an epitope of the protein env of the virus  
5 HCV.

2. An amino acid sequence according to claim 1, characterized in that it is comprised within the portion from the amino acid 209 to the amino acid 259 according to the numbering of Choo, Q.-L. et al.,  
10 Science (1988), 244:359362 (3).

3. An amino acid sequence according to claim 2, characterized in that it is comprised in the SEQ ID N1.

4. An amino acid sequence according to claim 3, characterized in that it comprises the portion from the  
15 amino acid 13 to the amino acid 46 of SEQ ID N1.

5. An amino acid sequence according to claim 3, characterized in that it comprises the portion from the amino acid 21 to the amino acid 30 of SEQ ID N1.

6. An amino acid sequence according to claim 2,  
20 characterized in that it is comprised in the SEQ ID N2.

7. An amino acid sequence according to claim 6, characterized in that it comprises the portion from the amino acid 13 to the amino acid 46 of the SEQ ID N2.

8. An amino acid sequence according to claim 6,  
25 characterized in that it comprises the portion from the amino acid 21 to the amino acid 30 of SEQ ID N2.

9. An amino acid sequence according to claim 2, characterized in that it is comprised within the SEQ ID N3.

5 10. An amino acid sequence according to claim 9, characterized in that it comprises the portion from the amino acid 13 to the amino acid 47 of SEQ ID N3.

11. An amino acid sequence according to claim 11, characterized in that it comprises the portion from the amino acid 21 to the amino acid 31 of SEQ ID N3.

10 12. Peptides characterized in that they have the amino acid sequence according to any one of the preceding claims.

13. Peptides according to claim 13 characterized in that they are synthetic peptides.

15 14. Peptides according to claim 12 or 13 characterized in that they have a conformational structure able to increase the immunogenicity thereof.

20 15. Peptides according to claim 14 characterized in that said conformational structure is achieved by reacting two residues of cysteine and by cyclizing the peptide.

16. A nucleotide sequence coding for an epitope of the env protein.

25 17. A nucleotide sequence according to claim 16, characterized in that it is comprised in the sequence SEQ ID N1.

18. A nucleotide sequence according to claim 16,  
characterized in that it is comprised in the sequence  
SEQ ID N2.

5 19. A nucleotide sequence according to claim 16,  
characterized in that it is comprised in the sequence  
SEQ ID N3.

20. A nucleotide sequence according to claim 19,  
characterized in that it comprises at least one  
fragment of 10 nucleotides of the SEQ ID N3.

10 21. A nucleotide sequence according to claim 16,  
characterized in that it comprises the sequence SEQ ID  
N3.

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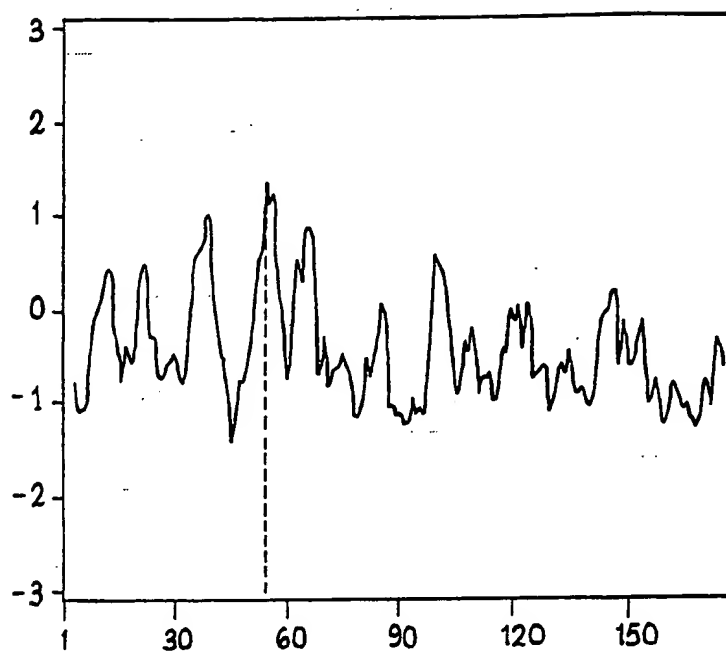


FIG. 1A

HYDROPHILIC PROFILE OF THE PROTEIC  
SEQUENCE HCVENV1, CALCULATED ON THE BASIS  
OF AN AVERAGE LENGTH OF 6 AMINOACIDS

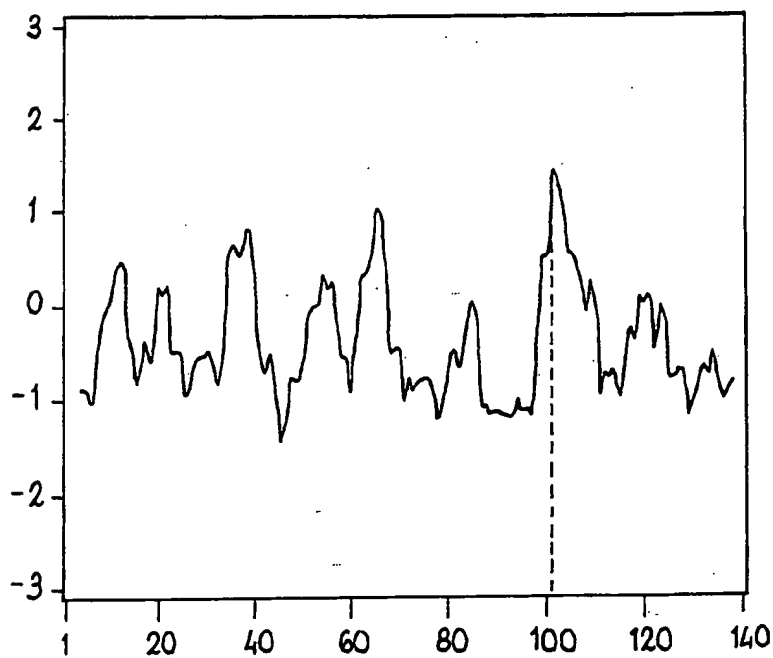


FIG. 1B

HYDROPHILIC PROFILE OF THE PROTEIC  
SEQUENCE HCVENV2, CALCULATED ON THE BASIS  
OF AN AVERAGE LENGTH OF 6 AMINOACIDS

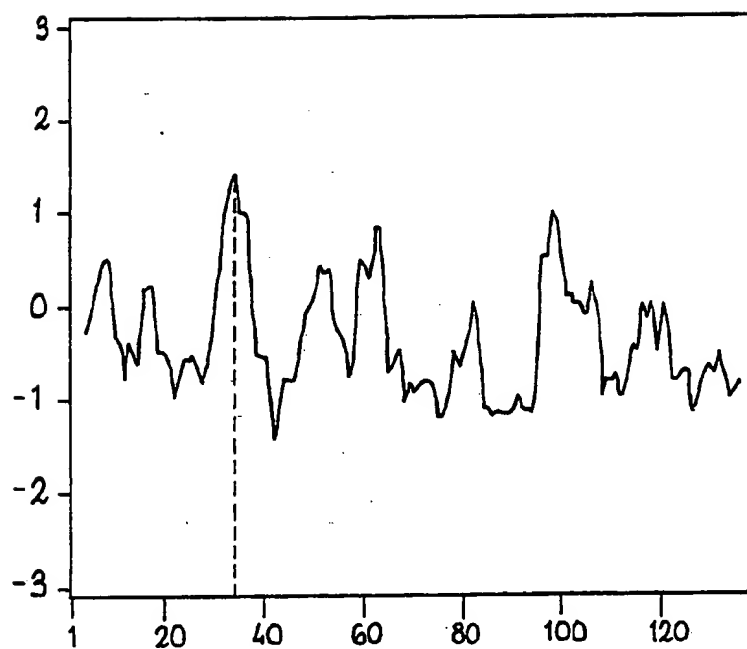


FIG. 1C

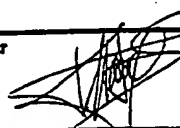
HYDROPHILIC PROFILE OF THE PROTEIC  
SEQUENCE HCVENV3, CALCULATED ON THE BASIS  
OF AN AVERAGE LENGTH OF 6 AMINOACIDS



## INTERNATIONAL SEARCH REPORT

PCT/IT 92/00081

International Application No

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C07K7/10;	C07K7/64;	A61K37/02; C07K15/00
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
O,X	PEPTIDE CHEMISTRY, 1991, OSAKA, JP & PROCEEDINGS OF THE 28TH SYMPOSIUM ON PEPTIDE CHEMISTRY, 27 October 1990, OSAKA, JP pages 211 - 214 MUNEKATA, E. ET AL. 'Epitope-mapping of hepatitis c constituting protein' see the whole document	1-2, 12-14, 16
O,Y	---	3-11, 17-21
X	WO,A,9 011 089 (CHIRON CORPORATION, USA) 4 October 1990 cited in the application	1-5, 12-14, 16,17
Y	see claims 1-14; figure 16 ---	6-11, 18-21
	---	-/--
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Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
29 OCTOBER 1992	16. 11. 92	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
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A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, April 1991, WASHINGTON US pages 3392 - 3396 OGATA, NORIO; ALTER, HARVEY J.; MILLER, ROGER H.; PURCELL, ROBERT H. 'Nucleotide sequence and mutation rate of the H strain of hepatitis C virus' -----	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
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